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## No Correlation Between Nasopharyngeal Human Bocavirus 1 Genome Load and mRNA Detection or Serology in Adeno-/Tonsillectomy Patients

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1 TITLE PAGE

2 Brief Report

3 Title: No Correlation Between Nasopharyngeal Human Bocavirus 1 Genome Load and mRNA  
4 Detection or Serology in Adeno-/tonsillectomy Patients

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6 Running title: HBoV1 in Nasopharynx and Tonsils

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31

32 Summary

33 Relatively high loads of HBoV1 DNA can be detected in the nasopharynx of asymptomatic

34 subjects, which are negative for mRNA and/or serodiagnostic markers. HBoV1 DNA quantitative

35 PCR may have lower specificity than HBoV1 mRNA detection for diagnosing symptomatic

36 infection.

37 FOOTNOTE PAGE

38 Conflict of interest

39 Dr. Allander has a patent Human bocavirus and methods of diagnosis and treatment licensed to  
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47

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51

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62    **ABSTRACT**

63    Human bocavirus 1 (HBoV1) can persist in nasopharynx and tonsils. Using HBoV1 serology,  
64    reverse-transcription polymerase chain reaction (PCR) for detecting messenger RNA (mRNA) and  
65    quantitative PCR for HBoV1 genome load count, we studied in what extent the HBoV1 DNA loads  
66    in nasopharynx correlates with acute infection markers. Tonsillar tissue, nasopharyngeal aspirate  
67    and serum were obtained from 188 elective adeno-/tonsillectomy patients. Relatively high loads of  
68    HBoV1 DNA were detected in the nasopharynx of 14 (7%) primarily asymptomatic subjects with  
69    negative mRNA and/or serodiagnostic results. Quantitative HBoV1 DNA PCR may have lower  
70    specificity than HBoV1 mRNA detection for diagnosing symptomatic infection.

71

72    Key words: bocavirus, parvovirus, nasopharynx, tonsil, serology, diagnosis, detection

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## 87 BACKGROUND

88 Human bocavirus (HBoV) was discovered in 2005 and belongs to the *Parvoviridae* family [1]. It is  
89 a non-enveloped single-stranded DNA virus causing mild to life-threatening respiratory tract  
90 infections in young children. HBoV1 is primarily transmitted by the respiratory route [1]. Three  
91 other human bocaviruses (HBoV2-4) have been discovered in stool and are considered enteric.  
92 HBoV1 is a frequent finding in young children suffering from lower respiratory tract illnesses such  
93 as bronchiolitis, wheezing, asthma and pneumonia [1,2]. The persistence of HBoV DNA in the  
94 airways and tonsils has been under investigation lately. In a recent study, HBoV DNA was found in  
95 tonsil squamous cell carcinoma tumors, prompting speculations of a possible causal association  
96 [3,4]. The virus is known to persist for weeks or months in the respiratory tract whereby a  
97 qualitative polymerase chain reaction (PCR) is insufficient as a diagnostic tool [1,2,5,6].  
98 Microbiological diagnosis is often incorrectly based on a qualitative multiplex PCR. The most  
99 reliable diagnosis of acute HBoV1 infection is considered to be based on messenger RNA (mRNA)  
100 or a high HBoV1 DNA load in nasopharyngeal aspirate (NPA), DNA in serum, and serology  
101 [1,2,5,7]. HBoV1 DNA has been shown to also persist in adenoids and tonsils of children [8]. The  
102 aim of this study was to evaluate if high HBoV1 DNA loads occur in NPA or tonsils in adeno-  
103 /tonsillectomy patients without acute HBoV1 infection, based on a documented lack of HBoV1  
104 mRNA and/or IgM, the gold standards for diagnosis. We hypothesized that there is no active  
105 bocavirus replication in persistent HBoV1 detection.

106

## 107 METHODS

### 108 Study Population

109 Tonsil and nasopharyngeal samples were collected from 200 consecutive patients who underwent  
110 adeno-/tonsillectomy at the Satakunta Central Hospital, Pori, Finland, between April 2008 and  
111 March 2009. The inclusion criteria were tonsillectomy, adenotonsillectomy or adenotomy due to

112 clinical indication and written informed approval from the study subject or his/her parents. Out of  
113 the 200 enrolled patients, 12 yielded low-quality samples. In total, 188 patients with a median age  
114 of 12 years (range 1-65) underwent elective adeno-/tonsillectomy (n=143) or sole adenotomy  
115 (n=45) and had sufficient and good quality biopsy samples for microbial and immunological studies  
116 [9]. The main indications for tonsillectomy were recurrent tonsillitis in 43 (30%) and tonsillar  
117 hypertrophy in 48 (34%) of 143 patients and for adenotonsillectomy, adenotonsillar hypertrophy in  
118 40 of 143 (28 %) patients, respectively [9]. Other indications (8%) for adeno-/tonsillectomy were  
119 e.g. throat abscess, recurrent fever, food remnants in tonsils and teeth braces. Indications for  
120 adenotomy were hypertrophy in 17 (38%) and recurrent otitis in 28 (62%) of 45 patients. All the  
121 study patients filled a standardized health questionnaire including respiratory symptoms 30 days  
122 before and after the operation [9]. On the operation day 127 (67%) had no respiratory tract  
123 symptoms, 37 (20%) reported mild respiratory symptoms and 24 (13%) had no data.

124

#### 125 Samples

126 Adeno-/tonsillectomy was performed by otorhinolaryngologists according to routine clinical  
127 procedure. A part of the internal tonsillar tissue was instantly cut in 3-4 mm cubes, stored in  
128 RNAlater, an RNA stabilization reagent (Qiagen, Hilden, Germany), incubated at +2-8 °C until the  
129 next working day and finally stored at -80 °C [9]. Nasopharyngeal aspirate samples were collected  
130 using a standardized procedure. If the aspirate yield was small, the collection was repeated after  
131 administration of 2 ml physiologic saline. For viral analyses, a part of the tonsils and a  
132 nasopharyngeal aspirate were stored in dry tubes at -80 °C [9]. The first sample of the paired serum  
133 samples was collected during the tonsillectomy anesthesia and the follow-up sample was taken in a  
134 median of 58 days (range 36-104).

135

#### 136 Ethical Approval

137 The study protocols were approved by the Ethics Committee of the Satakunta Central Hospital and  
138 by the Ethics Committee of the Hospital District of Southwest Finland.

139

#### 140 Virus Diagnostics

141 Virus diagnostics of all NPA and tonsil samples was performed according to clinical routine using  
142 PCR. Adenoid tissue samples were not analyzed. In-house real-time PCR assays were used to detect  
143 HBoV1, rhinovirus, enterovirus, and respiratory syncytial virus as described previously [9]. Seeplex  
144 RV12 ACE Detection (Seegene, Seoul, Korea) multiplex PCR assay was used for detection of  
145 adenovirus, coronaviruses (229E/NL63 and OC43/HKU1), influenza A and B viruses,  
146 metapneumovirus, parainfluenza virus types 1-3, respiratory syncytial virus group A and B, and  
147 rhinovirus according to manufacturer's instructions. Quantitative PCR (qPCR) was used for  
148 measuring the HBoV1 DNA load [10]. Serological tests for HBoV1-specific IgM and IgG were  
149 performed for 122 patients [5,11]. Serology of the adenotomy patients (n=45) was not analyzed. To  
150 verify that the IgG results were HBoV1 specific, the serum samples were blocked with HBoV2 and  
151 HBoV3 antigens. The mRNA expression levels of HBoV1 in NPA and tonsil samples were  
152 analyzed by reverse-transcription PCR (RT-PCR) [7]. An RT-PCR detecting human beta-actin  
153 mRNA was used as control for intactness of mRNA in the samples [12]. Virus PCR and qPCR were  
154 done at the Department of Virology, University of Turku, Turku, Finland, and at the Department of  
155 Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden. Serology was  
156 analyzed at the Department of Virology, University of Helsinki and the RT-PCR at the Norwegian  
157 University of Science and Technology, Trondheim, Norway.

158

#### 159 RESULTS

160 HBoV1 DNA in NPA, tonsillar tissue, or in both samples, could be detected in 40 patients (21%)  
161 with a median age of 5 years (range 1-22). These patients did not have severe respiratory tract



infection but 12 of 40 patients (30%) reported one or more of the following: mild rhinitis, cough, symptoms of otitis, throat pain or upper airway obstruction symptoms on the operation day. In the sole adenotomy group 8 of 15 patients (53%) and in the adeno-/tonsillectomy group 4 of 25 patients (16%), respectively, reported symptoms (Tables 1-2).

Twenty-eight patients were positive for HBoV1 DNA in NPA only, 7 in tonsillar tissue only and 5 in both samples. Five sole adenotomy patients had high ( $>10^6$  copies/ml) viral load in NPA using qPCR but were mRNA negative (Table 1). In the tonsillectomy group 9 patients had relatively high ( $>10^4$  copies/ml) viral load in NPA but were mRNA negative and corresponding sera available were HBoV1 IgM-negative (Table 2). Only 1 patient gave a (barely) IgM-positive test result, but with a stable IgG absorbance in paired samples (Table 2). In all but three patients, the HBoV1 DNA finding was accompanied with IgG positivity indicating a prior infection. These three HBoV1 DNA-positive but seronegative children had, however, prior HBoV2 immunity, which suggest that their HBoV1 IgG-negativity can be explained by an immunological phenomenon called original antigenic sin [13]. Furthermore, HBoV1-IgG levels did not increase in any of the 7 paired serum samples of HBoV1 DNA-positive patients (Table 2). All 29 NPAs and 8 tonsils analyzed were HBoV1-mRNA negative (Tables 1-2). Eight NPA samples with HBoV1 DNA loads  $>10^4$  were tested with the beta actin-mRNA PCR, all with strongly positive results.

## DISCUSSION

Our study confirms that HBoV1 can be found in the respiratory tract of patients with chronic and recurrent adenotonsillar disease. Quite a high prevalence (21%) of HBoV1 DNA in tonsils and/or NPA of elective adeno-/tonsillectomy patients was detected which agrees with earlier studies [8]. An even higher prevalence (43%) has been discovered in mainly asymptomatic subjects but the patients were small children (median age of 23 months) undergoing elective adeno-/tonsillectomy

187 and/or myringotomy [14]. We also found relatively high ( $>10^4$  copies/ml) or high ( $>10^6$  copies/ml)  
188 HBoV1 DNA loads in nasopharynx of 13% and 3% our study patients, respectively. However, the  
189 high DNA loads were not accompanied by positive HBoV1 mRNA or serological responses. Our  
190 results supported the study hypothesis that HBoV1 was not actively transcribing in persistent  
191 infection.

192  
193 The most common laboratory diagnostic method for respiratory infections is qualitative PCR,  
194 despite the fact that HBoV1 DNA can, due to prolonged presence or intermittent shedding, be  
195 detected in the nasopharynx for months after a symptomatic respiratory infection [1,6,15]. Previous  
196 studies have suggested that the DNA amount decreases over time and that high DNA loads ( $>10^4$  to  
197  $10^6$  copies/ml, depending on the study) would be a sign of acute bocavirus infection [2,5,7,15]. To  
198 define one specific threshold for high viral load is very demanding due to the various test methods,  
199 the type and quality of the specimens, and the time of collection. In our study we found high loads  
200 ( $>10^6$  copies/ml) of HBoV1 DNA particularly in adenotomy patients of which 3 were asymptomatic  
201 and 2 had mild respiratory tract symptoms. Only 1 adeno-/tonsillectomy patient with relatively high  
202 viral load ( $>10^4$  copies/ml) reported symptoms.

203  
204 In addition, mRNA of HBoV1 has been used as a marker of viral activity: HBoV1 mRNA can be  
205 detected in NPA of patients with symptomatic respiratory tract infection but not in asymptomatic  
206 controls [2,7]. It is known that HBoV1 DNA is stored in adenotonsillar tissue [8]. We wanted to  
207 investigate the viral activity in tonsils. None of the tonsils showed HBoV1 mRNA regardless of the  
208 HBoV-DNA load. Furthermore, all NPAs were also mRNA negative, in line with earlier studies of  
209 non-acute HBoV1 infections[7,8]. Conversely, in previous studies, the detection of HBoV1 mRNA  
210 in symptomatic patients was associated with high HBoV1 DNA loads [2,7]. In our elective adeno-  
211 /tonsillectomy patients, relatively high loads of HBoV1 DNA in the respiratory tract were not

212 associated with concomitant viral replication demonstrated by the lack of mRNA detection. Our  
213 data suggests that HBoV1 DNA or its high load by qPCR are less specific markers for acute  
214 HBoV1 infection than mRNA, at least in adenotonsillar surgery subjects. In this respect, our data  
215 support using HBoV1 mRNA detection as a more reliable method for diagnosing acute infection as  
216 suggested previously [2,7,8].  
217  
218 Serological results were in line with the clinical findings and did not support acute HBoV1  
219 infection in any patients. Since most patients studied by serology were  $\geq 5$  years of age, they most  
220 likely have already experienced primary bocavirus infection. The HBoV1 DNA finding in the  
221 respiratory tract was accompanied by IgG positivity in 18/25 cases (no sera available n=4), of  
222 which 17 were IgM negative, indicating past infections. The one barely IgM-positive patient with  
223 HBoV1 DNA in tonsils, showed an already high and stable IgG in paired samples, indicating a  
224 recent but non-acute infection. In two earlier studies among wheezing children, there has been an  
225 association of high ( $>10^4$  or  $>10^6$  copies/ml) HBoV1 DNA load with diagnostic serology [2,5].  
226 This association could not be found in the current study of primarily asymptomatic tonsillectomy  
227 patients due to lack of acute infections. We show that persisting HBoV1 DNA can be of relatively  
228 high loads also in non-acute infections.  
229  
230 This study provides new information about HBoV1 DNA positivity without clinical  
231 illness/manifestation and also confirms earlier results of HBoV1 diagnosis [2,5–7,15]. Earlier  
232 studies have focused on young children with respiratory tract infection [5,7,14,15] whereas our  
233 study had slightly older and mainly asymptomatic adeno- /tonsillectomy patients. A major  
234 limitation of the current study is that the data set was not complete: 8 of the 45 (18%) HBoV1 PCR-  
235 positive NPA or tonsillar tissue samples were not analyzed by mRNA RT-PCR. Another limitation  
236 of this study is the low number of paired serum samples. Serum samples were not available at the

237 enrollment (n=4), at the follow-up visit (n=10) or both samples (n=4). Serology of the adenotomy  
238 group was not analyzed. However, this is still the largest study on subjects without acute respiratory  
239 symptoms that compares different diagnostic methods for HBoV1 infection.

240

241 In conclusion, we did not find a correlation between HBoV1 genome load and mRNA detection or  
242 serology in adeno-/tonsillectomy patients. Our findings support the use of HBoV1 mRNA detection  
243 and serology as more specific diagnostic tools to identify acute bocavirus infection.

244

245 NOTES

246

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251

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254

255 Potential conflicts of interest

256 Dr. Allander has a patent Human bocavirus and methods of diagnosis and treatment licensed to  
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299

300

301 **Table 1. Adenotomy patients with HBoV1 DNA-positive NPA samples**

Case no.	Age (y)	Adenotomy indication	Symptoms <sup>a</sup> on the operation day	HBoV1 NPA PCR result	HBoV1 DNA load (cp/ml) in NPA	mRNA NPA
B013	4	ROM	yes	pos	128800	neg
B038	3	ROM	no	pos	123800	neg
B061	2	ROM	no	pos	550000	neg
B064	2	ROM	no	pos	176800	neg
B066	3	ROM	yes	pos	141200	neg
B073	3	ROM	no	pos	100396800	neg
B074	5	ROM	no	pos	36200	neg
B087	3	ROM	yes	pos	28269400	neg
B100	6	AH	yes	pos	19708800	neg
B122	8	AH	yes	pos	358200	neg
B126	4	AH	yes	pos	16800	neg
B129	2	ROM	yes	pos	117400	neg
B182	2	ROM	no	pos	20537600	neg
B184	1	ROM	no	pos	2227000	neg
B194	2	ROM	yes	pos	91600	neg

302 Abbreviations: y, years; ROM, recurrent otitis media; AH, adenoid hypertrophy; NPA,  
303 nasopharyngeal aspirate; cp, copies. <sup>a</sup>One or more of the following: mild rhinitis, cough, symptoms  
304 of otitis, throat pain, upper airway obstruction symptoms.

305 **Table 2. Adeno-/tonsillectomy patients with HBoV1 DNA-positive NPA and/or tonsillar tissue**  
306 **samples**

307

Case no.	Age (y)	Tonsillectomy indication	Symptoms <sup>a</sup> on the operation day	HBoV1 PCR result, NPA	HBoV1 DNA load (cp/ml), NPA	HBoV1 PCR result, tonsils
B004	6	ATH	no	pos	NA	neg
B008	6	ATH	no	pos	400	neg
B015	8	ATH	no	pos	500	neg
B021	8	ATH	no	pos	4400	neg
B028	16	RT, TH	no	pos	NA	neg
B051	7	ATH	no	pos	133200	neg
B069	8	RT	no	pos	600	neg
B113	12	ATH	NA	pos	119200	neg
B130	5	ROM, ATH	NA	pos	30400	neg
B160	7	ATH	yes	pos	4000	neg
B162	6	ATH	no	pos	210600	neg
B169	7	RT	no	pos	7600	neg
B185	7	ATH	NA	pos	8600	neg
B018	22	RT	yes	neg	0	pos
B019	5	ROM, RT, TH	no	neg	0	pos
B036	4	ATH	NA	neg	0	pos
B135	3	ATH	no	neg	0	pos
B193	2	ATH	no	neg	0	pos
B195	9	ATH	no	neg	0	pos



B198	3	ROM, ATH, recurrent fever	yes	neg	0	pos
B056	5	ATH	yes	pos	307800	pos
B082	4	ATH	no	pos	32200	pos
B106	4	ATH	no	pos	220800	pos
B150	5	RT, ATH	NA	pos	92400	pos
B197	3	ATH	no	pos	202600	pos

308

309 Abbreviations: y, years; ATH, adenotonsillar hypertrophy; TH, tonsillar hypertrophy, ROM,  
310 recurrent otitis media; RT, recurrent tonsillitis; NPA, nasopharyngeal aspirate; cp, copies; NA, not  
311 available; abs., absorbance (cutoff  $\geq 0.131$ ).

312 <sup>a</sup>One or more of the following: mild rhinitis, cough, symptoms of otitis, throat pain, upper airway  
313 obstruction symptoms.

314 <sup>b</sup>Paired serum samples; no increase in IgG.

315 <sup>c</sup>No acute-phase serum sample available.

316 <sup>d</sup>HBoV2 IgG positive; may influence induction of HBoV1 IgG through original antigenic sin [13].

317 <sup>e</sup>Very low absorbance level; 0,147. Together with a stable IgG level in paired samples, the  
318 interpretation is recent but non-acute HBoV1 infection.

319